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THE DEVELOPMENTAL APPEARANCE OF PATERNAL FORMS OF LACTATE DEHYDROGENASE AND MALATE DEHYDROGENASE IN HYBRID HORSESHOE CRABS[1]

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Abstract

Differences in electrophoretic mobilities of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) existed between three Asian horseshoe crabs, *Tachypleus tridentatus, Tachypleus gigas,* and *Carcinoscorpius rotundicauda,* used for interspecific hybridization. After electrophoresis of extracts of hybrid horseshoe crab embryos on starch gels, the paternal, maternal, and hybrid forms of the LDH and MDH were detected with specific enzyme staining. In viable hybrids the paternal form of the LDH was detected at stage 17 (immediately before the 1st embryonic molt). Similarly, evidence of gene expression for mitochondrial MDH was seen at stage 14 (stage of appearance of rudimental appendages). Gene expression for supernatant MDH was seen at stage 17 (immediately before the 1st embryonic molt). Regarding the onset of genome control in embryogenesis, it was suggested that prior to the activation of the maternal gene of the LDH, the paternal gene of the LDH was activated in horseshoe crab hybrids. Furthermore, there was evidence that the maternal effects on early embryogenesis were due to enzymes present in the egg prior to fertilization, not to continued synthesis directed by stable messenger RNA.

Introduction

Morphological studies on echinoderm, amphibian, teleost, and other species hybrids show that, in general, only maternal characters are evident until gastrular or postgastrular organogenesis. This conclusion is supported by many studies in which enzymes and other proteins of paternal type are first observed at postgastrular stages (Davidson, 1976). If two species with differences in specific enzymes form viable hybrids, and meternal- and paternal-type enzymes can be distinguished in the offspring, the paternal enzymes should not appear until after the new diploid genome is activated in the embryo. Therefore, the viable hybrids offer an opportunity for studying maternal and paternal contributions to development. To detect the paternal form of the enzyme, techniques of zone electrophoresis and specific enzyme staining have been applied to lactate dehydrogenase (LDH) in hybrids of frogs (Wright and Moyer, 1966, 1968; Wright and Subtelny, 1971) and fishes (Hitzeroth *et al.,* 1968; Goldberg *et al.,* 1969), and to malate dehydrogenase (MDH) in frog hybrids (Wright and Subtelny, 1971). In interspecific hybrids of arthoropods, however, no work has been carried out to detect the paternal forms of enzymes during the development of the embryo.

In this paper we report the time of the expression of the paternal genes controlling

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Abbreviations: LDH = lactate dehydrogenase; MDH = malate dehydrogenase.

lactate dehydrogenase and malate dehydrogenase in hybrid embryos of Asian horseshoe crabs.

Materials and Methods

The Japanese horseshoe crab, *Tachypleus tridentatus,* was collected from Imari and Fukuoka, Japan, and the Southeast Asian horseshoe crabs, *Tachypleus gigas* and *Carcinoscorpius rotundicauda,* were collected from the vicinity of Bangsaen, Thailand, by Professor Smarn Srithunya (Zoological Museum and Marine Aquarium, Srinakharinwirot University, Thailand).

To contrast the paternal influence of three horseshoe crabs, eggs obtained from one female were divided into three groups, and each group was artificially inseminated by sperm from one of the three species and kept at 30°C. Cross-fertilizations were made in all nine combinations among three Asian horseshoe crab species. The developmental stage of the embryos was determined according to the normal plate of the Japanese horseshoe crab, *T. tridentatus,* described by Sekiguchi (1973), because fertilized eggs of *T. gigas, C. rotundicauda,* and the interspecific hybrids developed into swimming larvae (the first-instar larvae) through a similar morphological process to those of *T. tridentatus.*

A single embryo at each stage was homogenized in one or two drops of distilled water. Larval extracts were prepared from a single animal at the first-instar stage (just after hatching). To prepare the adult tissue extracts, the hepatopancreas was homogenized in a volume of distilled water approximately equal to the tissue volume, because all isozyme molecules of the LDH were included in horseshoe crab hepatopancreas. Sample homogenates were absorbed on a small piece of Toyo No. 50 filter paper and inserted into slits cut in the starch gel. Electrophoresis was carried out at 4°C with 11% or 12% gel horizontally for embryonic samples or vertically for larval and adult samples. Horizontal gel electrophoresis for embryonic and larval MDH was carried out using Davis' (1964) buffer system. Selander and Yang's (1969) buffer system was used during vertical gel electrophoresis for larval and adult MDH. Gel and electrode buffers for LDH isozymes were prepared according to the method of Selander and Yang (1969). A 100 ml staining mixture for the LDH consisted of 0.025 *M* Tris-HCl buffer (pH 7.4), 50 mg nicotinamide adenine dinucleotide, 35 mg nitro blue tetrazolium, 3 mg phenazine methosulphate, 2.0 ml 60% Na lactate, and 1.0 ml 0.5 *M* KCN (Shows and Ruddle, 1968). The staining mixture for the MDH was identical to the LDH but 10 ml 1.0 *M* Na malate, pH 7.0, was substituted for 2.0 ml 60% Na lactate.

Results

Lactate dehydrogenase

Before we consider the developing enzyme patterns in hybrids, we must examine whether the enzyme variants are present in adult and larval samples. Figure 1 shows the electrophoretic patterns of the LDH from the hepatopancreas tissues and the first-instar larvae of 3 Asian horseshoe crabs. The larval LDH from 3 species showed only one enzymic band, while the LDH from the hepatopancreas tissues of *T. tridentatus* and *C. rotundicauda* occurred in 3 isozymic forms on starch gel. Furthermore, the LDH from each of interspecific hybrid larvae showed 3 enzymic bands, suggesting that 2 peptides produced from paternal and maternal genes for the LDH could form heterodimers in horseshoe crab hybrids (Fig. 1B). The adult LDH of *T. tridentatus* and *C. rotundicauda* was monomorphic and that of *T. gigas*

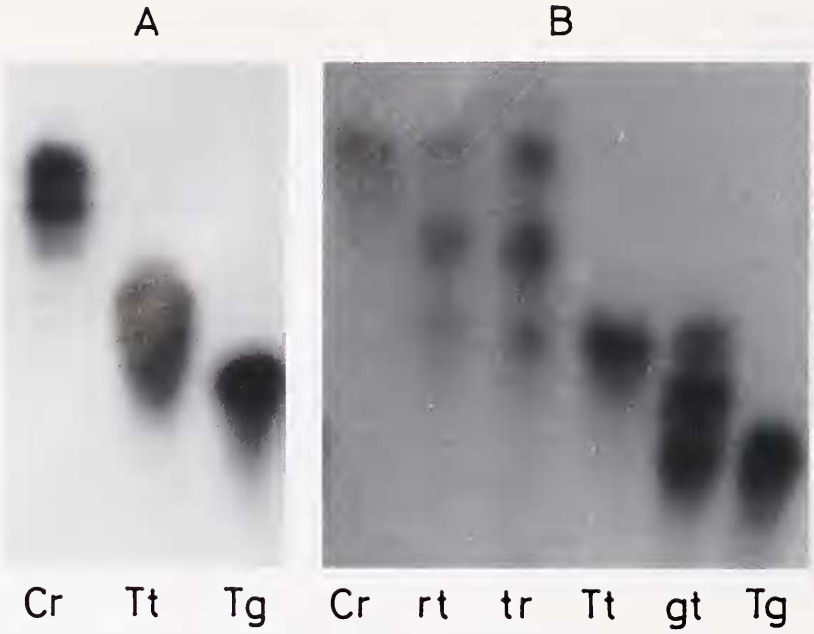

FIGURE 1. Electrophoretic patterns of horseshoe crab LDH from hepatopancreas tissues (A) and from the first-instar larvae (B). Vertical starch gel electrophoresis was carried out at 4°C with 12% gel, using the buffer system of Selander and Yang (1969. Gel buffer: 0.08 *M* Tris and 0.005 *M* citric acid, pH 8.7. Electrode buffer: 0.3 *M* boric acid and 0.06 *M* NaOH, pH 8.2). Cr = *Carcinoscorpius rotundicauda;* Tt = *Tachypleus tridentatus;* Tg = *Tachypleus gigas;* rt = hybrid between Cr ♀ and Tt ♂; tr = hybrid between Tt ♀ and Cr ♂; gt = hybrid between Tg ♀ and Tt ♂.

was polymorphic (Sugita and Sekiguchi, in prep.). Genetic variants of the LDH could not be detected in larvae developed from eggs of a single female.

In hybrid progeny obtained from the interspecific crosses of all 6 combinations among 3 species as well as in normal progeny from the control crosses, the early embryos displayed only the maternal LDH pattern which could be detected in unfertilized eggs (results not shown). The maternal LDH from these embryos had similar relative mobility to the LDH from the first-instar larvae (Fig. 1B).

In hybrid embryos between *T. tridentatus* ♀ and *C. rotundicauda* ♂, the paternal form of the LDH was first detected at stage 17 (30 days after insemination, immediately before the 1st embryonic molt) (Fig. 2A), but the paternal form of the LDH was not observed even on the 41st day after insemination (stage 19, after the the 2nd embryonic molt) in hybrid embryos between *C. rotundicauda* ♀ and *T. tridentatus* ♂.

The LDH from hybrid embryo between *T. gigas* ♀ and *T. tridentatus* ♂ occurred in 3 molecular forms at stage 20 (32 days after insemination, after the 3rd embryonic molt), suggesting that the LDH of the hybrid embryo consisted of a maternal homodimer, a paternal homodimer, and a hybrid heterodimer (Fig. 2B). This paternal form of the enzyme was first observed at stage 18 (28 days after insemination, after the 1st embryonic molt) in hybrid embryo *T. gigas* ♀ × *T. tridentatus* ♂ (results not shown). On the other hand, the LDH from *T. tridentatus* ♀ × *T. gigas* ♂ hybrid embryo showed only the maternal form on the 32nd day after insemination (Fig.

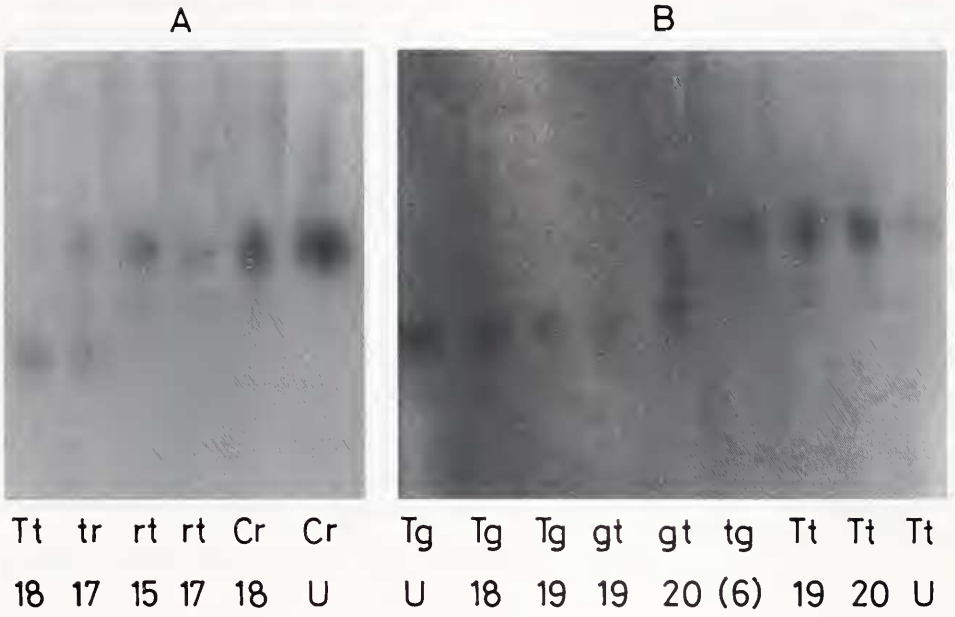

FIGURE 2. Electrophoretic patterns of the LDH in unfertilized eggs and developing embryos of 3 Asian horseshoe crabs and their hybrids. The hybridization experiments were carried out 2 times using different sets of 3 pairs (3 species) of horseshoe crabs. Electrophoretic patterns of the LDH in the 30th-day and 32nd-day embryos from the 2 experiments are shown separately in A and B, except for a column Cr U in A. Horizontal starch gel electrophoresis was carried out at 4°C with 11% gel, using the same buffer system as explained in Figure 1. Cr, Tg, Tt, gt, rt, and tr are as described in Figure 1. tg = Hybrid between Tt ♀ and Tg ♂; U = unfertilized egg. Unfertilized eggs as well as fertilized eggs were cultured in sea water at 30°C for 3 days (A) and 32 days (B). Numbers indicate the developmental stage of Sekiguchi's normal plate (Sekiguchi, 1973). The number 6 in parentheses means that hybrid embryo used was able to live on until the 32nd day after insemination, although the development had stopped at stage 6 (blastula stage).

2B). Until this day the hybrid was able to live on, although the development had stopped at blastula stage, or stage 6 (Sekiguchi and Sugita, 1980; Sugita *et al.*, 1982).

The hybridized eggs of *C. rotundicauda* ♀ × *T. gigas* ♂ and the reciprocal cross stopped their development at blastula stage (Sekiguchi and Sugita, 1980; Sugita *et al.*, 1982) and never expressed the paternal forms of the LDH (results not shown).

Malate dehydrogenase

There are 2 major electrophoretic forms of the MDH in the horseshoe crab, *Limulus polyphemus,* as well as in most animals and higher plants. These isozymes are controlled by separate genetic loci and are localized in different subcellular fractions, a mitochondrial form and a supernatant form (Selander *et al.*, 1970). On a gel run with Davis' (1964) buffer system, the larval MDH from 3 Asian horseshoe crabs showed the slower-migrating system (MDH-1), which was the mitochondrial form, and the faster-migrating system (MDH-2), or the supernatant form as Selander *et al.* (1970) reported with *Limulus* MDH using the buffer system of Selander and Yang (1969). When, in our laboratory, electrophoresis was carried out using Selander and Yang's (1969) buffer system, the mitochondrial bands were very close to the

supernatant bands on a gel. Therefore, we used Davis' (1964) buffer system to examine the developing MDH patterns in Asian horseshoe crabs and their hybrids.

There were electrophoretic variants of the MDH in 3 Asian horseshoe crabs, but genetic variants of the MDH were not detected in larvae developed from eggs of a single female (compare the MDH-1 of columns Tg, gt, and gr in Fig. 3 with that of columns Tg and gt in Fig. 4).

The early embryos displayed only the maternal forms of both MDH-1 and MDH-2 in hybrid and normal progeny and these enzyme forms were detected in unfertilized eggs of 3 species (Fig. 3). The paternal form of the MDH-1 was first detected in the *T. tridentatus* ♀ × *C. rotundicauda* ♂ embryo at stage 14 (stage of appearance of rudimental appendages, 28 days after insemination) (Fig. 4A), while in the hybrid embryo of the reciprocal cross the paternal forms of the MDH-1 and MDH-2 were not expressed even on the 41st day after insemination (stage 19, after the 2nd embryonic molt).

The paternal form of the MDH-2 was displayed in the *T. gigas* ♀ × *T. tridentatus* ♂ embryos at stage 19 (after the 2nd embryonic molt) and stage 20 (after the 3rd embryonic molt) (Fig. 4B). This paternal form in the *T. gigas* ♀ × *T. tridentatus* ♂ embryo was first observed at stage 17 (22 days after insemination, immediately before the 1st embryonic molt, results not shown).

On the other hand, the hybrid embryos whose development was stopped at blastula stage, that is, *T. tridentatus* ♀ × *T. gigas* ♂, *T. gigas* ♀ × *C. rotundicauda*

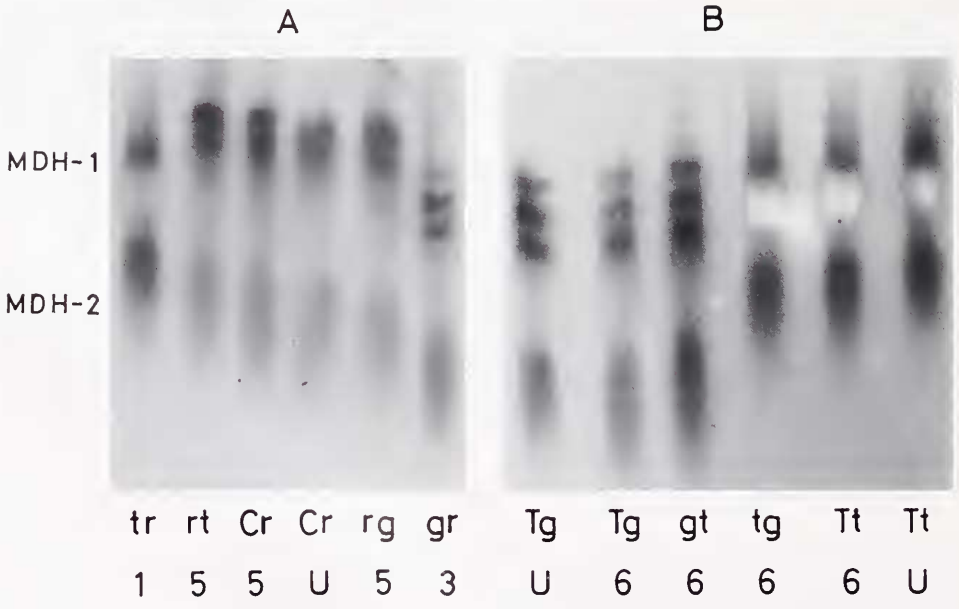

FIGURE 3. Electrophoretic patterns of the MDH in unfertilized eggs and early embryos of 3 Asian horseshoe crabs and their hybrids. The 3rd-day and 6th-day embryos from a hybridization experiment were used in A and B, respectively. Horizontal starch gel electrophoresis was carried out at 4°C with 11% gel, using the buffer system of Davis (1964. Gel buffer: 0.38 *M* Tris-HCl, pH 8.9. Electrode buffer: 0.005 *M* Tris and 0.038 *M* glycine, pH 8.3). Numbers indicate the developmental stage of Sekiguchi's normal plate (Sekiguchi, 1973). Symbols are explained in Figures 1 and 2, except for symbols defined below. MDH-1 = Slower-migrating system, or mitochondrial form; MDH-2 = faster-migrating system, or supernatant form; gr = hybrid between Tg ♀ and Cr ♂; rg = hybrid between Cr ♀ and Tg ♂.

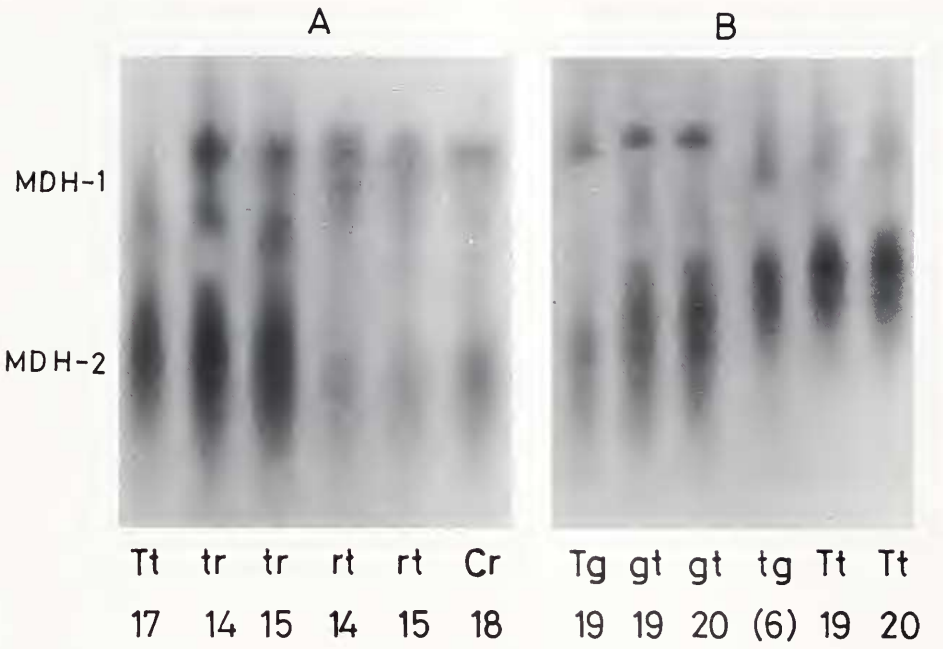

FIGURE 4. Electrophoretic patterns of the MDH from the 28th-day (A) and 32nd-day (B) embryos of 3 Asian horseshoe crabs and their hybrids. Electrophoretic patterns of the MDH in embryos obtained from different sets of 3 pairs (3 species) of horseshoe crabs are shown separately in A and B. Horizontal starch gel electrophoresis was carried out at 4°C with 11% gel using the same buffer system as in Figure 3. Numbers indicate the developmental stage of Sekiguchi's normal plate (Sekiguchi, 1973). The number 6 in parentheses means that the hybrid embryo was able to live on until the 32nd day after insemination, although the development had stopped at stage 6 (blastula stage). All symbols are explained in Figures 1, 2, and 3.

♂, and *C. rotundicauda* ♀ × *T. gigas* ♂ embryos did not express the paternal forms of the MDH-1 and MDH-2.

DISCUSSION

The LDH of horseshoe crabs is D-lactate specific and has a molecular weight of approximately 70,000 (Long and Kaplan, 1968, 1973). This D-LDH occurs in 3 dimeric forms, not in 5 tetrameric forms as does the L-LDH of vertebrates with a molecular weight of 140,000 (Selander and Yang, 1970; see columns Tt and Cr in Fig. 1A). Although each LDH from the larvae of 3 Asian horseshoe crabs shows only one dimeric form with different electrophoretic mobility from one another, the LDH from the first-instar larvae of hybrid horseshoe crabs is composed of 3 molecular forms: a maternal homodimer, a paternal homodimer, and a hybrid heterodimer (Fig. 1B). This hybrid LDH heterodimer was detected with maternal and paternal homodimers in *T. gigas* ♀ × *T. tridentatus* ♂ embryo at stage 20 (Fig. 2B). However, the paternal LDH homodimer from this cross-fertilized embryo was first observed without the hybrid heterodimer at stage 18 (results not shown). Similarly, in *T. tridentatus* ♀ × *C. rotundicauda* ♂ embryo the paternal homodimer of the LDH was first detected at stage 17 (Fig. 2A) without the hybrid heterodimer which was observed at stage 19 (41 days after insemination, results not shown).

Based on the findings that no hybrid enzymes were detected in androgenetic haploid frog hybrids, Wright and Subtelny (1971) indicated that the degradation of maternal (cytoplasmic) enzymes *in vivo* did not yield subunits capable of reaggregation with newly synthesized subunits to form active enzymes. This means that the hybrid forms of enzymes are expressed at the time when both maternal and paternal genes for the enzymes are activated together. Therefore, the findings that the paternal and maternal homodimers were detected without their hybrid heterodimer indicate that, with regard to the onset of genome control in embryogenesis, prior to the activation of the maternal gene of the LDH the paternal gene of the LDH was activated in the horseshoe crab hybrids.

The time of expression of the paternal genes controlling the mitochondrial malate dehydrogenase (MDH-1) and supernatant malate dehydrogenase (MDH-2) was examined, although they did not show clear, electrophoretic patterns. Evidence of paternal gene expression for the MDH-1 was seen in *T. tridentatus* ♀ × *C. rotundicauda* ♂ embryos at stage 14 (Fig. 4A). Expression of paternal gene for the MDH-2 was seen in *T. gigas* ♀ × *T. tridentatus* ♂ embryos at stage 17 (results not shown).

In early embryos only the maternal forms of the LDH and MDH were observed until postgastrular organogenesis (stage 13, stage of the germ-band formation). The active maternal forms of these enzymes were present in unfertilized eggs of 3 Asian horseshoe crabs (Figs. 2, 3) and the steady state activity of the maternal enzymes in unfertilized eggs did not change dramatically during the culture for 32 days at 30°C in sea water (Fig. 2B). These and other results present evidence that the maternal effects on early embryogenesis are due to enzymes present in the egg prior to fertilization, not to continued synthesis directed by stable messenger RNA (Wright and Subtelny, 1971).

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